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Assessing the Role of Polyethylene Glycol (PEG) in Improving Functional Recovery Following Spinal Cord Injury

Dustin Dalton

Virginia Commonwealth University

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**Assessing the Role of Polyethylene Glycol (PEG) in Improving Functional Recovery
Following Spinal Cord Injury**

A thesis in partial fulfillment of the requirements for the degree of Master of Science in Anatomy
and Neurobiology at Virginia Commonwealth University

By:

Dustin Kelley Dalton

B.S., University of North Carolina at Chapel Hill, 2008

Director Raymond J. Colello, D.Phil

Associate Professor, Department of Anatomy and Neurobiology

Virginia Commonwealth University

Richmond, Virginia

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List of Abbreviations

3D	Three Dimensional
ChABC	Chondroitinase ABC
CNS	Central Nervous System
DRG	Dorsal Root Ganglion
EM	Electron Microscopy
HFIP	Hexa-fluoro-isopropanol
NGF	Nerve Growth Factor
PDS	Polydioxanone Suture
PGLA	Polyglycolic-Lactic Acid
SEM	Scanning Electron Micrograph
SCI	Spinal Cord Injury
TEM	Transmission Electron Micrograph

ABSTRACT

ASSESSING THE ROLE OF PEG IN IMPROVING FUNCTIONAL RECOVERY
FOLLOWING SPINAL CORD INJURY

Dustin Kelley Dalton, Master of Science

A thesis in partial fulfillment of the requirements for the degree of Master of Science in Anatomy
at Virginia Commonwealth University

Virginia Commonwealth University, 2011

Raymond J. Colello, D.Phil., Associate Professor, Department of Anatomy and Neurobiology

Injury to the spinal cord results in the disruption of signal transmission between the brain and distal targets. It often presents with the loss of motor function and sensory perception below the level of injury. There are many obstacles following injury that must be overcome in order to encourage axon regeneration and improve functional recovery. A combinatorial approach is necessary to combat physical and chemical barriers to recovery. The fluid filled cyst that forms in the majority of spinal cord injuries presents a physical barrier that we treat with our electrospun bridges. We implanted our bridges into female Long Evans Hooded rats following a complete transection. Using a molecular fusogen, polyethylene glycol, known to seal damaged membranes in conjunction with our bridges, we were able to increase functional recovery compared to animals treated with a bridge and saline. In Chapter 1, we introduce spinal cord anatomy, the pathological classifications, axon pathology, and our therapeutic strategy. Chapter 2 details the materials and methods. Chapter 3 examines previous uses of polyethylene glycol as a molecular fusogen, previous studies utilizing it in spinal cord injury, and our strategy of fusing damaged axons to improve functional recovery. Finally in Chapter 4, I discuss our behavioral results, compare histology, and detail the future of our research including protocol improvements and future combination therapies that include PEG to improve outcome.

Chapter 1

Introduction to Spinal Cord Injury: Anatomy, Axon Pathology, and Bridging the Gap

Synopsis:

What is Spinal Cord Injury?

Injury to the spinal cord, typically due to blunt or penetrating trauma, results in the disruption of signal transmission between the brain and distal targets and typically presents with functional loss below the lesion. The extent of functional deficits depends on the completeness of the injury. Often a combination of sensation and mobility below the injury are compromised or completely lost. Spinal cord injury is caused by a primary event, and exacerbated by secondary cellular and biochemical events which result in additional damage to the spinal cord and reduce the potential for regeneration and recovery. Traumatic injury to axons, including membrane breaches or complete axon severing, can lead to the intracellular release of death signals driving the neurons to apoptosis. In addition, inflammatory cells infiltrate the lesion area and release toxic substrates, which drive viable neurons and glia surviving the initial trauma into apoptosis. The axons that undergo apoptosis following injury leave cytoskeletal and myelin debris, inhibiting axon regrowth from the damaged but surviving neurons. Glial cells bordering the injury contribute to secondary injury, becoming reactive and proliferating to form a barrier or glial scar. Reactive glia also upregulate their production of molecules that inhibit axon growth such as chondroitin sulfate proteoglycans. The glial cell scar forms rostral and caudal to the site

of injury and the damaged parenchyma succumbs to liquifactive necrosis. The end result is a fluid filled cyst which acts as a physical gap further complicating regeneration.

The events described above are only a few of the many challenges faced in understanding and treating spinal cord injury. A result of the complexity of spinal cord injury is that a single therapeutic strategy has not been successful. Treatment strategies involving a combination of therapies are the future of spinal cord injury treatment. This dissertation examines the behavioral improvements and histology of implanting a 3D electrospun matrix used in combination with polyethylene glycol, a well-known membrane fusogen, in a complete transection model of spinal cord injury. First we will discuss spinal cord anatomy, injury classifications, and axon pathology. In the later chapters we will discuss molecular fusogens and our ideas for promoting axon survival as well as our experimental results.

Introduction:

Functional Anatomy of the Spinal Cord

The human spinal cord is a soft, long, ovoid-shaped bundle of nervous tissue protected by the bony vertebral column. The cord runs from the level of the foramen magnum to the upper lumbar vertebrae and contains over a billion neurons transmitting information between the brain and peripheral targets. The spinal cord is encased in the same three meninges covering the brain; the outermost dura mater, the arachnoid mater, and the innermost pia mater. White matter is found in the cord periphery and contains ascending and descending axon tracts that act as signal channels relaying information between the body and the brain. Gray matter is centrally located and contains neuronal cell bodies, interneuron connections, central pattern generators, and reflex

loops. The dorsal aspect of the spinal cord generally contains afferent, or sensory, axon tracts, and the ventral aspect of the spinal cord generally contains efferent, or motor, axon tracts.

The areas of spinal cord most susceptible to injury are the cervical and lumbar regions; this is due to the anatomy of the spinal cord in these areas. The circumference of the cervical and lumbar spinal cord increases as a result of limb innervation and the space between the cord and bony vertebrae decreases. In the reduced physical space, the cervical and lumbar enlargements can be easily compressed during a traumatic injury.

Pathological Classification

Trauma to the spinal cord can be caused by a single event, or a combination of stresses on the cord such as squeezing, stretching, bruising, crushing or tearing. A pathological classification relating the injury to the spinal cord with the histological appearance of the cord seen during autopsies was created, narrowing injuries into four groups. The classification groups include solid cord injuries, contusion/cavitation injuries, laceration injuries, and massive compression injuries (Bunge et al, 1993).

Solid Cord Injuries

About 10% of all SCI cases are solid cord injuries. The cord grossly appears normal. No bruising, discoloration, or softening of the cord is seen on the outside, and the meninges are intact. Histological analysis, however, reveals a loss of spinal cord architecture in the injured tissue (Figure 1.1B). The cause of solid cord injuries is unclear. It is thought that a slight application of pressure such as stretching or squeezing, which itself would not cause severe injury, triggers an inflammatory cascade. Inflammation, a key secondary injury component, is

believed to be a major contributor to tissue damage in solid cord injuries (Norenberg, J. Smith, & Marcillo, 2004).

Contusion/Cavitation Injuries

The most prevalent variation of traumatic SCI is the contusion/cavitation injury, which represents more than half of all cases. These injuries are usually caused by displaced discs and ligaments or dislocated vertebra that compress and bruise the cord. There is often bleeding within the central grey matter during these injuries, however, the meninges remain intact and there is no disruption of the pial surface. Liquefactive necrosis eventually takes place, and the remaining cellular debris is cleared by inflammatory cells leaving a fluid-filled cyst (Figure 1.1C). Reactive astrogliosis occurs alongside necrosis; astrocytes proliferate and hypertrophy forming a gliotic scar surrounding the cyst (Norenberg, J. Smith, & Marcillo, 2004).

Laceration Injuries

Laceration injuries are most commonly the result of fractured vertebrae cutting the cord, but may also occur during stabbings or gunshot wounds. Approximately 20% of SCI injuries are laceration injuries. In laceration injuries, unlike contusion injuries, there is a disruption in the meninges resulting in the migration of collagen depositing fibroblasts into the lesion (Figure 1.1D). Fibroblasts eventually create a mesenchymal scar, and patient prognosis is poor due to dense scarring that blocks axonal regeneration or rewiring (Norenberg, J. Smith, & Marcillo, 2004).

Massive Compression Injuries

Massive compression injuries (Figure 1.1E) represent 20% of traumatic SCI cases. In these injuries a significant force completely crushes the cord. Like laceration injuries, the disruption of the meninges leads to significant scar formation, fibrosis, and dural adhesions; all of which are additional obstacles to recovery (Norenberg, J. Smith, & Marcillo, 2004).

Clinical Classification

The clinical classification of any SCI is determined by the functional deficits post-injury. Clinically injuries can be complete, incomplete, or discomplete. Complete injuries usually result in complete paralysis and loss of function below the level of the injury. Patients with incomplete injuries retain or recover some function below the level of injury, implying that some axon tracts survived the insult. Discomplete injuries appear clinically complete but neurophysiologically incomplete. Patients with this type of injury exhibit some conscious influence on the spinal cord. Discomplete injuries are not fully understood.

SCI Overview

After a primary injury consisting of mechanical disruption of spinal cord tissues, a variety of secondary events exacerbate cell death. Inflammation occurs over the first few hours to days following injury to the spinal cord and can increase apoptosis and make the damage initially sustained worse (Dusart and Schwab, 1994; Blight, 1992; Blight et al., 1995). In addition to inflammation around the injury site, reactive gliosis occurs in which surrounding glial cells undergo hypertrophy and hyperplasia forming a dense gliotic scar. This scar acts as a physical barrier to axon growth (Reier et al., 1983). Reactive astrocytes composing the gliotic scar also release molecules that inhibit neuron growth creating a biochemical barrier to axon regeneration

(McKeon, Schreiber, Rudge, & Silver, 1991). As secondary injury progresses, liquefactive necrosis of cells takes place leaving a fluid filled cyst and a physical gap between the surviving severed axons and their targets (Figure 1.2).

Axon Pathology

Following a traumatic injury, many axons that become severed, either by the initial insult or by secondary injury, degenerate. The distal end undergoes Wallerian degeneration which is characterized by fragmenting and unraveling myelin sheaths and the disintegration of axons that have been detached from their cell body (Norenberg, J. Smith, & Marcillo, 2004).

Pathologically, the endoplasmic reticulum breaks down, the neurofilaments degrade, the mitochondria swell, and the distal end of the axon breaks up into fragments that are phagocytized (Griffin, George, & Chaudhry, 1996). This degeneration occurs over a protracted period, taking months to complete, and it can produce reactive astrocytes that reduce neurite outgrowth (Bovolenta et al., 1992).

The proximal end of a severed axon experiences an initial, rapid dieback retreating toward the soma (Figure 1.3B, 1.3C). This early axonal dieback accounts for the majority of total retraction distance experienced by the axon during the first days after trauma (Kerschensteiner, Schwab, Lichtman, & Misgeld, 2005). At the tips of severed axons, bulbous swellings form following an injury due to the soma continuing to deliver cytoskeletal proteins and organelles to the distal end via anterograde transport (Figure 1.3A) (Büki, Povlishock, 2006). Rupture of these bulbous ends contribute to further damage of the surrounding nervous tissue, and the retraction of the axons may decrease their ability to respond to treatments administered at the site of injury (Hagg, Oudega, 2006)

A breach in the neuron cell membrane is the earliest physical manifestation of spinal cord injury at the cellular level. The compromised barrier allows ions to flow down their concentration gradients, disrupting cytoskeletal architecture, and depolarizing local areas of the neuron causing a conduction block (Maxwell, 1996) (Dimitrijevic, 1995). Conduction block results in the loss of signal transmission in intact or damaged axons producing physical symptoms such as the loss of sensation and/or mobility. Unrestricted ion flow results in an influx of calcium (Iwata et al., 2004) which injures the mitochondria and causes calpain activation which plays a role in apoptosis and necrosis (Wingrave et al., 2003). Axon membrane damage also results in the release of cytochrome c and activation of caspases which further damage the neuron and drive the cell toward apoptosis (Bao and Liu, 2003). The mechanical damage, and ultimately the rate of cell death, is dependent on many factors including the magnitude of the insult. Many cells surviving the initial insult experience secondary injury causing a delayed but progressive death. We propose that if membrane breaches can be sealed quickly and permanently, damage to neurons will be reduced, and the total number of cells surviving a traumatic injury will be increased.

Therapeutic Strategy

As previously described, contusion injuries are the most common and leave a fluid filled cyst following liquefactive necrosis. This cavity represents a physical gap axons must overcome in order for functional recovery to be possible. Many bridging materials have been tested previously. A few examples include; support cells (Xu et al., 1995; Li et al., 1997), stem cells (McDonald et al., 1999; Akiyama et al., 2002), embryonic grafts (Reier et al., 1986) autologous grafts (von Wild and Brunelli, 2003; Houle et al., 2006), and synthetic substances (Tsai et al., 2004; Wen and Tresco, 2006). Many of these methods have shown some axon growth on to the

bridge, there has been little success in axons completing the journey across the bridge to the tissue on the other side.

We believe there are several criteria a bridge must meet in order to be a possible therapeutic option. A bridge should be biocompatible, three dimensional, fill the entire cavity, and porous enough for cell infiltration. The bridge should also allow the delivery of molecules or compounds designed to promote axon regeneration and neuroprotection. Our method of two-pole air gap electrospinning creates a bridge that meets all of these criteria. Air gap electrospinning uses an electric field between two grounded posts. Fibers whip back and forth between the grounded posts creating a three dimensional, cylindrical scaffold with individual fibers aligned along the length of the bridge (Jha et al., 2010). The air gap electrospinning apparatus is shown in Figure 1.4. Electrospun fibers can be composed of a variety of substances from extracellular matrix proteins to synthetic polymers, and properties of the final structures such as porosity, alignment, and fiber diameter can be adjusted (Fridrikh et al., 2003). Electrospinning has been used in tissue engineering for dermal implants or synthetic vessels (Stizel et al., 2001; Sun et al., 2005). We chose to use the synthetic polymer polydioxanone suture (PDS) in our spinal cord injury studies. PDS is a resorbable suture material, it is biocompatible and FDA approved for other uses, and it has a lifespan *in vivo* of approximately 6 months (Molea et al, 2000; Boland et al., 2005).

In previous work we have demonstrated how our electrospun bridge is porous enough for cell infiltration. An image from a Scanning Electron Microscope shows the structure of the fibers and porosity of the matrix (Figures 1.5A and 1.5B). We have also shown that neurite growth can be directed by the aligned fibers of the bridge. In an *in vitro* experiment, we planted

dorsal root ganglions (DRG) taken from neonatal rats on our bridges and noted the directional outgrowth mimics the direction of the bridge's fibers (Figure 1.6) (Chow et al., 2007).

Our previous *in vivo* work with rats has shown some limited success. After implanting an electrospun bridges in to the spinal cord and supplementing it with trophic support in the form of nerve growth factor (NGF) and Chondroitinase ABC (ChABC) which breaks down inhibitory compounds released by reactive astrocytes, we see an increase in behavioral recovery when compared with animals that did not receive trophic support or animals that did not receive an implant (Fig. 1.7).

We have demonstrated the benefit of using our air gap electrospun bridge. We now hypothesize that using our bridge in conjunction with a well-known membrane fusogen to rapidly seal damaged membrane breaches we could increase the number of neurons that survive a traumatic injury. By increasing neuron survival, we expect to see an increase in functional recovery.

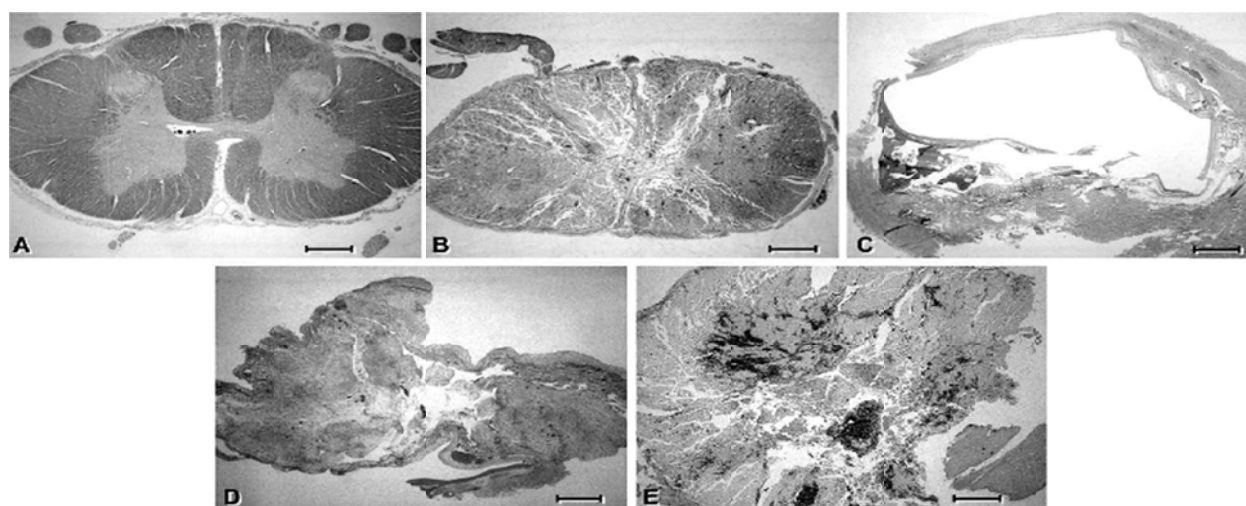


Figure 1.1 Pathology of the Spinal Cord. (A) Cervical cross section of a normal human spinal cord. (B) Loss of internal spinal cord architecture seen in solid cord injuries. (C) Formation of a fluid filled cyst seen in contusion/cavitation injuries. (D) Disruption of the pia in laceration injuries. (E) Loss of tissue and hemorrhage in massive compression injuries. Scale Bars (A to E) = 2 mm. (Norenberg et al., 2004)

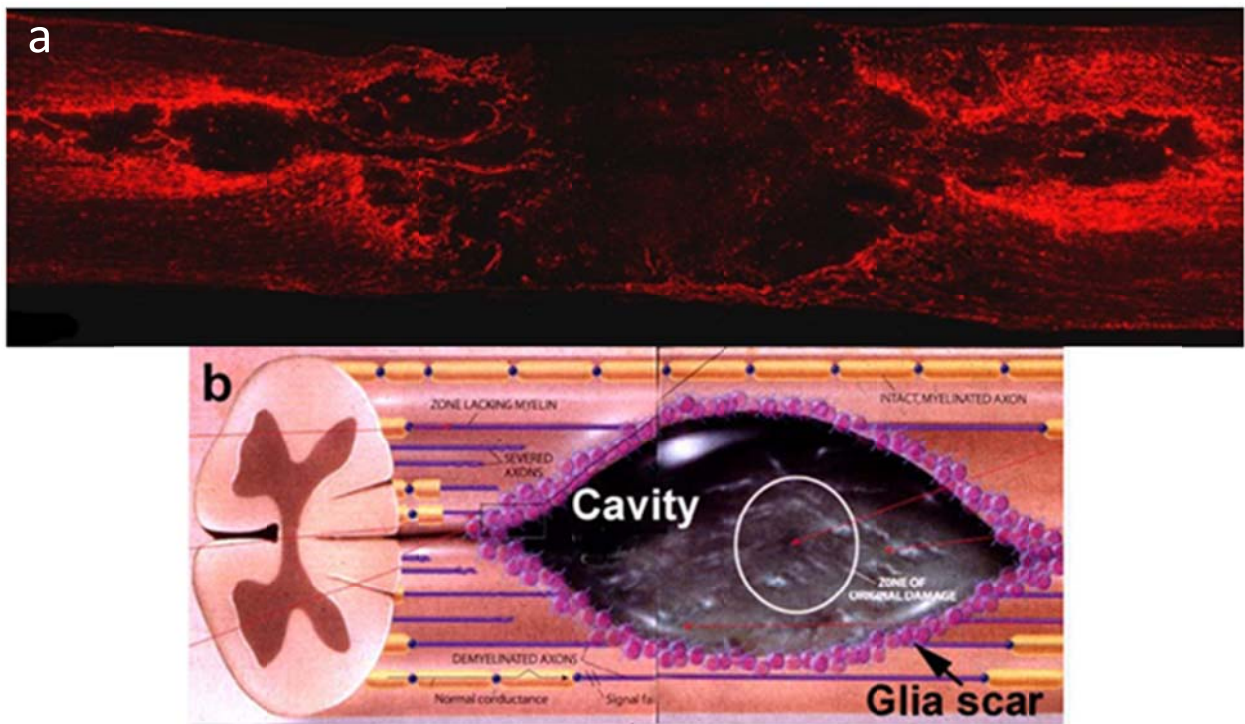


Figure 1.2 The Cystic Cavity. (A) Longitudinal sections of a rat spinal cord, following a contusion injury, immunolabeled for GFAP (red). Notice the large central cavity that forms following trauma (Chow, 2009). (B) A schematic of the glial scar and cavity that form post injury. Figure 1B was modified from McDonald et al., (1999).

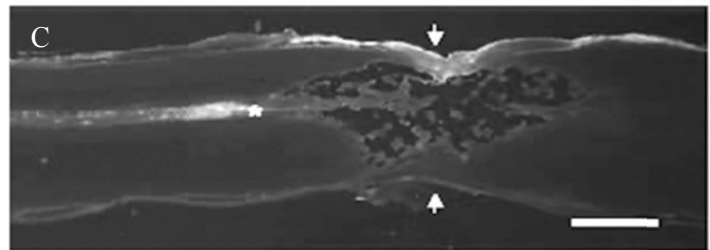
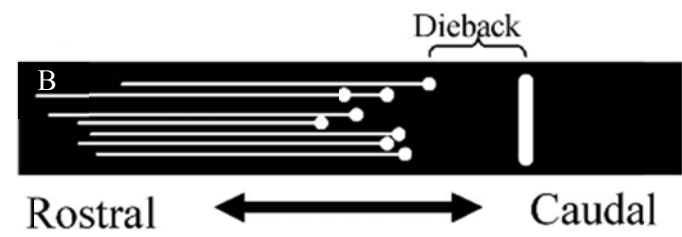
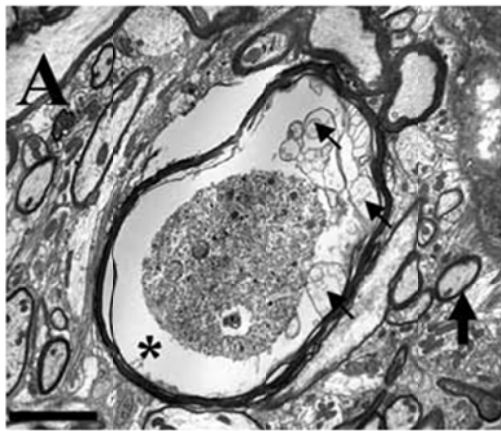


Figure 1.3 Axon Pathology. (A) Electron micrograph of a terminal bulb at 5000x. The diameter of the terminal bulb is 10 microns as opposed to the 1 micron diameter of normal axons (thick arrow). Degenerating intracellular debris can be seen in the middle of the swollen structure. The myelin is separated from the axon due to axonal swelling. Scale bar 2 microns. (B) Schematic of axon dieback from the lesion represented by the white bar (C) Axon dieback following injury visualized in parasagittal sections of fluorescently labeled corticospinal tract. White arrows represent injury epicenter. Scale bar = 1mm. Figure modified from (Seif, Nomura, & Tator, 2007).

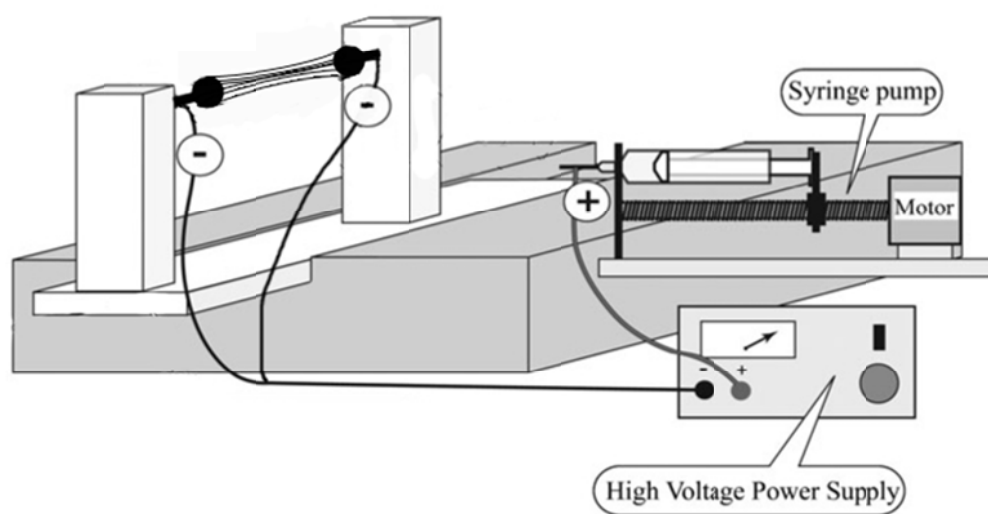


Figure 1.4 The Airgap Electrospinning Apparatus. Key system components include a solution reservoir (syringe), nozzle (18-gauge, blunted, metallic needle), high-voltage DC power supply, and grounded target (rotating mandrel). The bridge forms as a 3-Dimensional cylinder between the two grounded posts.

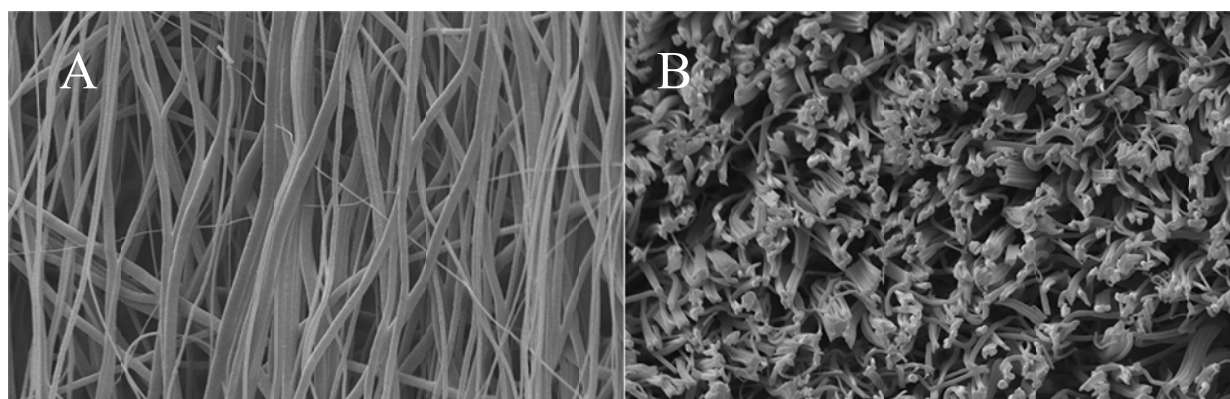


Figure 1.5 SEM of PDS Matrix Monofilaments. (A) Scanning Electron Micrograph at 2000x showing aligned fibers in a representative bridge. (B) SEM at 3000x taken of a PDS bridge cut in cross section. Notice how the fibers are densely packed but maintain the porosity for cells and axons to grow through.

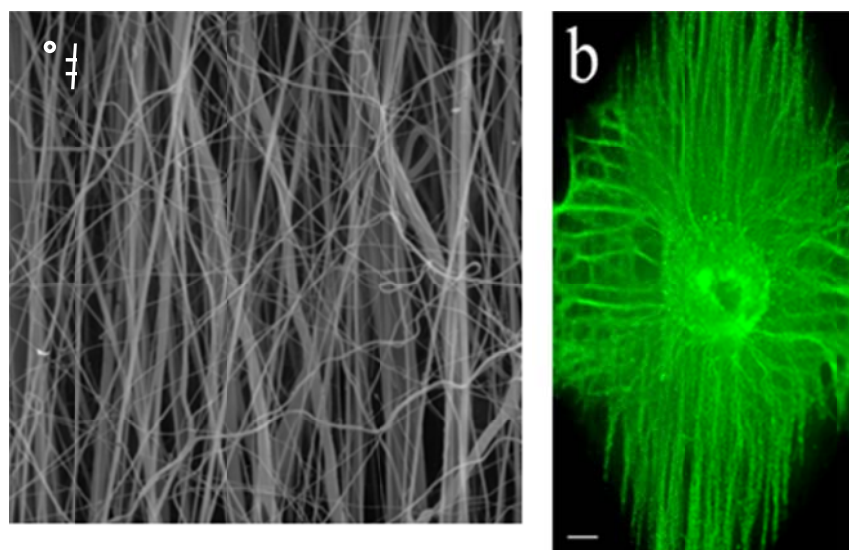


Figure 1.6 Electrospun Bridge Directs Neurite Growth. (A) An aligned bridge (B) Neurites stained with Tuj1 display a directional growth that mimic the fiber alignment of the bridge. Scale bar = 200 μm .

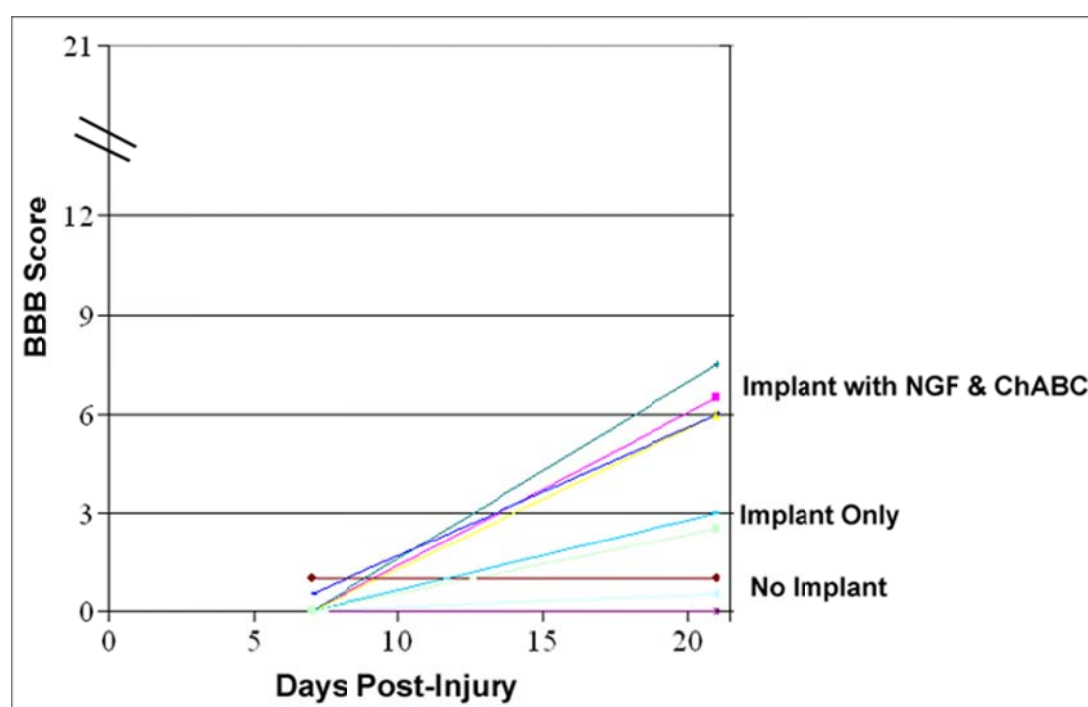


Figure 1.7 Previous Work Showing Success With Our Bridge. Graph displaying the BBB scores at varying times post-surgery of animals that have received either no bridge, an aligned bridge or an aligned bridge with trophic support (NGF and ChABC beads). There are noticeable differences emerging after 3 weeks. We want to further increase this functional recovery by increasing the number of cells surviving a traumatic injury. The BBB open field test assesses hind-limb mobility and is scored on a 21 point system; 0 is complete hind limb paralysis and 21 is complete mobility.

Chapter 2:

Materials and Methods

Air Gap Electrospinning:

PDS II or Polydioxanone monofilament surgical suture (Ethicon) is weighed out and dissolved in the solvent 1,1,1,3,3,3 hexafluoro-2-propanol (Sigma-Aldrich) at a concentration of 100mg/ml. The solution is gently shaken overnight to facilitate suture dissolving. Once dissolved, the solution is transferred to a 5-ml syringe capped with a blunted 18-gauge needle, and the syringe is mounted in a syringe pump (KD Scientific 100). A conductive circular washer is placed over the needle tip, connected to a high voltage DC power supply (Spellman CZE1000R) with an alligator clip, and positively charged to 20kV. Two grounded, vertical poles placed 5 to 10 cm apart are used to contain the negative field between two metallic bullets oriented inward and perpendicular to the vertical poles. Individual fibers are deposited across the negative field in series of parallel loops between the bullets. This 2 pole, air gap electrospinning setup produces cylindrical scaffolds constructed of millions individual fibers oriented to the long axis of the implant.

PGLA Coating:

Once the scaffold reaches the approximate diameter of the thoracic region of the rat spinal cord, it is cut down from the electrospinning poles and placed flat on a circular 120mm diameter steel plate. A coating of Polyglycolic-Lactic Acid (PGLA) (Sigma) composed of 50% polyglycolic acid and 50% polylactic acid is dissolved the in the same way as PDS, placed in a syringe and sprayed through a positively charged needle. The 2 pole system is removed, the

steel plate is negatively charged, and the PGLA is deposited on the surface of the PDS nerve guide which is rotated to ensure an even coat.

Scaffold Cutting:

To cut the bridge in to equal 1mm thick discs for implantation a rat brain blocker (David Kopf Instruments) was used. The portion of the construct most uniform in shape and thickness is selected for implantation and cut into 6 to 8mm segments. Those segments are individually placed in the brain blocker, held stationary by razor blades at either end and sectioned in to 1mm thick discs by slicing razor blades through the gaps in the block. This creates several 1mm discs out of the large uncut cylinder, the best of which are used for implantation.

Application of PEG:

Immediately prior to implantation, the 1mm bridges are completely saturated in a solution of polyethylene glycol (Sigma) with a molecular weight of 1500 g/mol for 2 minutes. They are then gently removed from the dish and inserted into the severed spinal cord. Saline was substituted for control animals.

Animal Model and Surgical Procedure:

Animals were acquired from Harlan Inc. The animals used were 100-120 day old female Long Evans Hooded rats. All surgical and postoperative care procedures were performed in accordance with the Virginia Commonwealth University Institutional Animal Care and Use Committee. Animals were anesthetized in a closed container with 5.0% Isoflurane (AERANE) and quickly shaved in a separate area to maintain aseptic conditions at the operating table. Animals were then placed in an anesthesia nosecone (Parkland Scientific) attached to a

stereotaxic table and maintained under 2.5% Isoflurane for the duration of the surgery. Depth of anesthesia was periodically measured by watching for a reflex response after a tail pinch. Ocular ointment was used to prevent dehydration of the eyes during the operation, and a warming blanket was placed underneath the animals to maintain body temperature. The shaved skin was cleaned with betadyne, and using sterile techniques, a dorsal midline incision was made over the mid-thoracic spine through the skin. Two additional incisions were made lateral to the spinal column to separate the back muscles from the spinous processes of the vertebrae. Tissue spreaders were then used to increase exposure of the vertebrae, and T8 was confirmed by palpation of the back. Using rongeurs, a thoracic laminectomy was performed to expose the spinal cord. A transection was made using micro scissors (FST) in a single cut fashion spreading the scissors over the entire cord and cutting through. If unsure that the entire cord was transected, a second cut was made in the same place. A 1mm PDS electrospun disc was then inserted into the spinal cord at the site of the transection. Following implantation, muscular layers were closed and sutured together with silk (Ethicon), and the skin was stapled shut with Mitchell staples (FST). Antibacterial cream (Bacitril) was applied to the incision. Two groups of three animals and two time points were used in this thesis. A saline control or a polyethylene glycol saturated bridge was inserted into the transected spinal cord, and the animals survived for one or two months.

Post-Operative Care:

Following surgery, rats were placed on a heating pad to maintain body temperature and monitored until conscious. Once awake they were given an injection of gentamicin (10mg/kg) and buprenorphine (0.5mg/kg). In the days following, buprenorphine was administered every 12 hours for the first 48 hours and daily prophylactic doses of gentamicin was given for 7 days after

surgery. Acetaminophen (150mg/kg) was added to the water for 3 days post-op. Urine was manually voided by palpitation of the bladder three times a day until the rats began to self micturate (urine volume measured <2ml/day). Urinary tract infections were diagnosed by clarity and smell of urine and treated with a 5 day cycle of gentamicin.

Basso-Beattie-Bresnahan (BBB) Locomotor Rating Scale:

The BBB locomotor rating scale is a well-known animal behavior test to assess hindlimb mobility. The rats were assessed and scored on a 21-point system with 21 being complete mobility and 0 being complete hindlimb paralysis (Table 2.1). The test rates parameters such as joint mobility, weight support, limb coordination, and gait stability. To perform the BBB test, animals were placed in an open field (large tabletop surround by a high barrier) and allowed to ambulate to the degree they are able for a four minute time period. The animals were evaluated weekly for the duration of the experiments (1 to 8 weeks). Animals were evaluated prior to surgery for four minutes in an open field to ensure they did not suffer from any motor deficits. Animals were scored on the BBB scale by two observers and if the scores differed they were averaged together.

Tissue Collection:

The rats were euthanized using Euthasol (150mg/kg), a pentobarbital agent, injected into the intraperitoneal space to induce a general plane of anesthesia. They were then perfused transcardially with 0.1 M phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde and 0.25% glutaraldehyde solution for electron microscopy. Following the perfusion, spinal cords were dissected out and placed in the fixation solution for 48 hours at 4°C. A 5 millimeter section of the cord including the lesion area was dissected out and embedded in 4% agar in distilled water. The embedded tissue was mounted on a chuck and cut into 100µm thin coronal

sections using the vibratome. Sections were cut completely through the lesion, and the tissue was collected in 48 well plates with fixative before being prepped for EM.

Electron Microscopy:

The 100 μ m thin sections were placed in 1% OsO₄ for 1 hour, dehydrated through an ethanol series, stained with uranyl acetate for 1 hour, and infiltrated overnight in a 1:1 propylene oxide and epon/araldite solution. The following day sections were placed in 100% epon/araldite for 1 hour then embedded and placed in the 60 degree oven for 2 days. The sections were then remounted on to plastic stubs for cutting and 1 μ m semi-thin sections were obtained and stained with toluidine blue. Coronal thin sections were also cut to view the tissue under the transmission electron microscope.

Statistical Analysis

The statistical data presented in this thesis is reported as means \pm standard error of the mean. Statistical analysis was performed using the software package Sigma Plot. Open field BBB scores were analyzed by t-test. Differences with a p value less than 0.05 were considered statistically significant.

BBB Locomotor Rating Scale

0	No observable hind limb movement
1	Slight movement of one or two joints, usually the hip and/or knee
2	Extensive movement of one joint or extensive movement of one joint and slight movement of one other joint
3	Extensive movement of two joints
4	Slight movement of all three joints of the hind limb
5	Slight movement of two joints and extensive movement of the third
6	Extensive movement of two joints and slight movement of the third
7	Extensive movement of all three joints of the hind limb
8	Sweeping with no weight support or plantar placement of the paw with no weight support
9	Plantar placement of the paw with weight support in stance only (i.e., when stationary) or occasional, frequent, or consistent weight supported dorsal stepping and no plantar stepping
10	Occasional weight supported plantar steps, no forelimb-hind limb coordination
11	Frequent to consistent weight supported plantar steps and no forelimb-hind limb coordination
12	Frequent to consistent weight supported plantar steps and occasional forelimb-hind limb coordination
13	Frequent to consistent weight supported plantar steps and frequent forelimb-hind limb coordination
14	Consistent weight supported plantar steps, consistent forelimb-hind limb coordination, and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance or frequent plantar stepping, consistent forelimb-hind limb coordination, and occasional dorsal stepping
15	Consistent plantar stepping and consistent forelimb-hind limb coordination; and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact
16	Consistent plantar stepping and consistent forelimb-hind limb coordination during gait; and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
17	Consistent plantar stepping and consistent forelimb-hind limb coordination during gait; and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and lift off
18	Consistent plantar stepping and consistent forelimb-hind limb coordination during gait; and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
19	Consistent plantar stepping and consistent forelimb-hind limb coordination during gait; and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and lift off; and tail is down part or all of the time
20	Consistent plantar stepping and consistent coordinated gait; consistent toe clearance; predominant paw position is parallel at initial contact and lift off; tail consistently up; and trunk instability
21	Consistent plantar stepping and coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, consistent trunk stability, tail consistently up

Terms and Operational Definitions

Slight	partial joint movement through less than half the range of joint motion
Extensive	movement through more than half of the range of joint motion
Sweeping	rhythmic movement of hind limb in which all three joints are extended, then fully flex and extend again; animal is usually lying on its side, the plantar surface of paw may or may not contact the ground; no weight support across the hind limb is evident
No Weight Support	no contraction of the extensor muscles of the hind limb during plantar placement of the paw; or no elevation of the hindquarter
Weight Support	contraction of the extensor muscles of the hind limb during plantar placement of the paw, or elevation of the hindquarter
Plantar Stepping	paw is in plantar contact with weight support then the hind limb is advanced forward and plantar contact with weight support is reestablished
Dorsal Stepping	weight is supported through the dorsal surface of the paw at some point in the step cycle
forelimb-hind limb	for every forelimb step an hind limb step is taken and the hind limbs alternate
Coordination	
Occasional	less than or equal to half; <50%
Frequent	more than half but not always; 51-94%
Consistent	nearly always or always; 95-100%
Trunk Instability	lateral weight shifts that cause waddling from side to side or a partial collapse of the trunk

Table 2.1 The 21-point BBB locomotor scale used to rate functional recovery after SCI.
(Adapted from Basso et al., 1995)

Chapter 3:

Fusogens: PEG

Introduction:

A molecule that aids in the sealing of membranes and can be used in the joining two cells together or to seal damaged membranes is called a fusogen. There are a few known fusogens including Polyethylene Glycol or PEG (Davidson et al., 1976), EPAN 680 which is a co-polymer of ethylene oxide-propylene and oxide-ethylene oxide (Nakajime et al., 1994) and Cephalin, a lipid formally named phosphatidylethanolamine (Golestani et al., 2007). In our work we used Polyethylene glycol (PEG) which has been used for decades as a fusogen in the formation of hybridomas for antibody production. Previous work has shown that sealing membrane breaches by a chemical fusogen can restore ion gradients (Luo, Borgens, & Shi, 2004) and reduce cell death (Luo & Shi, 2007). Knowing that the rupture of an axon membrane causes severe damage to the neuron in a traumatic spinal cord injury, application of a fusogen could be a possible therapeutic intervention following SCI to seal damaged membranes and improve recovery.

What is PEG?

PEG is a hydrophilic polymer that has the chemical formula is $\text{H}(\text{OCH}_2\text{CH}_2)_n\text{OH}$, its molecular weight depends on the number of repeating subunit, and it ranges from a colorless viscous liquid to a waxy solid depending on its molecular weight. PEG is an FDA approved pharmacological agent, it is a common solvent for injectable drugs, and it is frequently a base for ointments and lotions. In addition, PEG is also used in the fusion of cell membranes in the formation of hybridomas (Davidson, O'Malley, Wheeler, 1976, Lentz, 1994). The evidence of

the fusogenic capabilities of PEG made it the strongest candidate to test our hypothesis that sealing axons would facilitate functional recovery following SCI.

PEG Whole Cell Fusion

Polyethylene glycol long been known to fuse whole cells together. As early as 1975, it was shown to fuse erythrocytes together (Ahkong et al., 1975). For more than thirty years, PEG has been used in the fusion of antibody producing B-cells with immortal myeloma cells in the formation of hybridomas (Figure 3.1) (Davidson, O'Malley, Wheeler, 1976). It has also been used to fuse many neuron-like PC-12 cells together to produce a large multinucleated single unit to test neurophysiological measurements *in vitro* (Figure 3.2) (O'Lague, Huttner, 1980).

PEG Damaged Membrane Fusion

In addition to fusing whole cells together, PEG has also been shown to repair damaged nerve cell membranes. When horseradish peroxidase (HRP) is added to the media of an injured spinal cord, it penetrates the membrane of injured axons. Following further processing with a substrate that becomes oxidized by HRP, typically diaminobenzidine (DAB), the cells that have taken up HRP become visible. The lab of Dr. Richard Borgens at Perdue demonstrated a significant reduction in the uptake of the intracellular marker HRP with the addition of PEG following a crush injury (Figure 3.3) (Cho, Shi, Borgens, Ivanisevic, 2008). In addition to sealing crushed axons, PEG has also been shown to seal transected axons *in vitro* using dorsal root ganglion (DRG) cells grown in culture. Neurite projections extending out from DRGs were transected, PEG was applied to the media, and at varying times after the cells were washed in solution containing a fluorescent marker. Cells were determined to be sealed if their fluorescence was equal or less intense than neighboring, uninjured cells (Figure 3.4A). The

percentage of cells sealed with PEG and excluding the dye was much larger than that of those cells not sealed with PEG (Figure 3.4B). This experiment is especially important because it shows that PEG has the capacity to seal membrane breaches the size of axon diameters (Nehrt, Hamann, Ouyang, Shi, 2010).

PEG has also been shown to fuse closely apposed end of severed axons in less than a minute (Krause, Bittner, 1990). After severing the medial giant axon of an earthworm, Bittner applied saline (Figure 3.5A) as a control and PEG (Figure 3.5B) to test the ability of PEG to fuse the axon ends back together. Within sixty seconds greater than 80% of the closely apposed PEG treated giant axons were fused back together. This shows the speed with which PEG can seal damaged axon membranes. For our work, speed is important because the faster a damaged membrane can be resealed, the less damage it will experience, and the more likely it is for the cell to survive.

PEG Decreases Apoptosis

Following SCI, apoptosis is a major source of cell loss, and is believed to contribute to neurological deficits (Beattie, Farooqui, Bresnahan, 2000; Lu, Ashwell, Waite, 2000). It has previously been shown that PEG can significantly reduce secondary injury caused by oxidative stress (Luo, Borgens, Shi, 2004). Oxidative stress is important in regulating apoptosis (Carmody, Cotter, 2001; Raha, Robinson, 2001; Wood, Youle, 1995), and the reduction of this stress has been attributed to PEG mediated mitochondria protection (Luo, Borgens, Shi, 2004). Using the TUNEL staining technique, an application of PEG following a compression injury reduced apoptosis at both one day and seven days post injury (Figure 3.6) (Luo, Shi, 2007). To detect cells undergoing apoptosis the terminal deoxynucleotidyltransferase-mediated dUTP end

labeling (TUNEL) methodology was used which labels fragmented DNA frequently found in apoptotic cells (Gavrieli et al., 1992; Kaufmann et al., 2000). Demonstrating that PEG reduces apoptosis following spinal cord injury is crucial find for our study. By applying PEG to our treatments we hypothesize that cell survival would increase and an improvement in functional recovery would follow.

Proposed Mechanism of PEG Neuroprotection

A traumatic spinal cord injury creates breaches in neuronal cell membranes. Applying PEG can seal these breaches and does so rapidly. It is hypothesized that where breaches occur, PEG forms a protective barrier spanning the holes. PEG acts in a volume-exclusion manner dehydrating the local membrane areas and allowing the membrane components adjacent to the breach to move, rejoin, and seal the disruption (Lentz, 2007; Luo, Shi, 2007). Some PEG molecules may also enter the injured cell through breaches and interact with stressed mitochondrial membranes preventing swelling and rupture (Luo, Shi, 2007). A diagram of the proposed mechanism is shown in Figure 3.7.

PEG in Spinal Cord Injury

Thus far we have discussed the merits of PEG. It has been shown to fuse whole cells to form hybridomas (Davidson, O'Malley, Wheeler, 1976), fuse damaged membranes back together (Cho, Shi, Borgens, Ivanisevic, 2008), fuse completely severed axons back together (Krause, Bittner, 1990), and reduce apoptosis following spinal cord injury (Luo, Shi, 2007). Given the success of PEG, we proposed to use it in conjunction with our PDS bridge following complete transection of the spinal cord. We anticipate PEG will seal damaged axon membranes, and possibly increase functional recovery following injury.

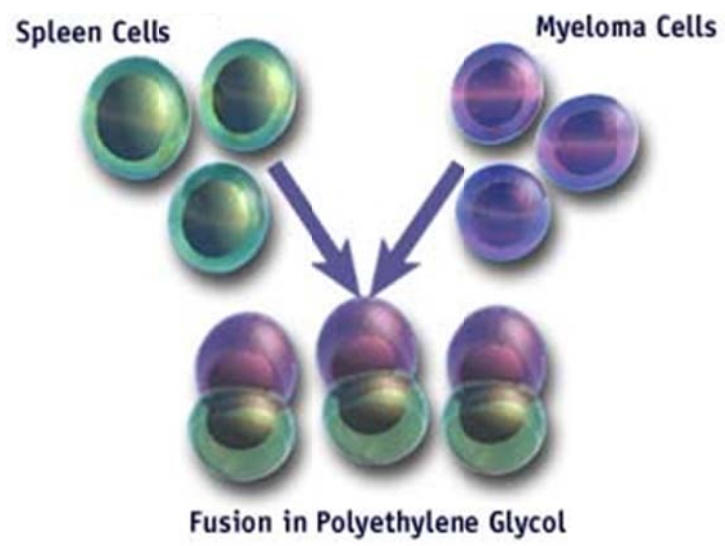


Figure 3.1 Hybridoma Formation. PEG has been used for more than 30 years in the formation of hybridomas, fusing lymphocytes with an immortal myeloma cell line for antibody production. Figure from http://www.hopkins-arthritis.org/physician-corner/education/biomedical-science/mono_anti.html

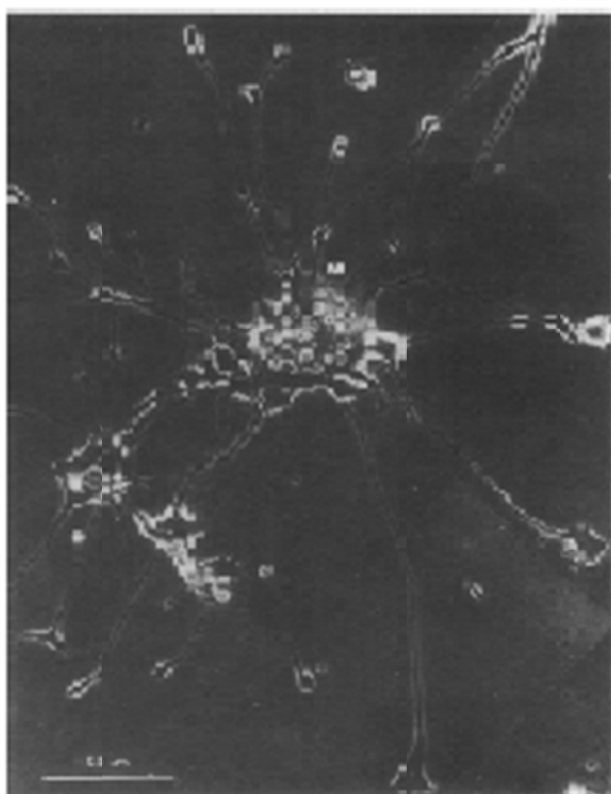


Figure 3.2 PEG Fusing Whole Cells. PEG has been shown to fuse many pheochromocytoma cells (PC-12 cells, neuron-like cells) together to produce large single units. These large, multinucleated cells are used in facilitating neurophysiological measurements in vitro. Figure from O'Lague & Huttner, 1980.

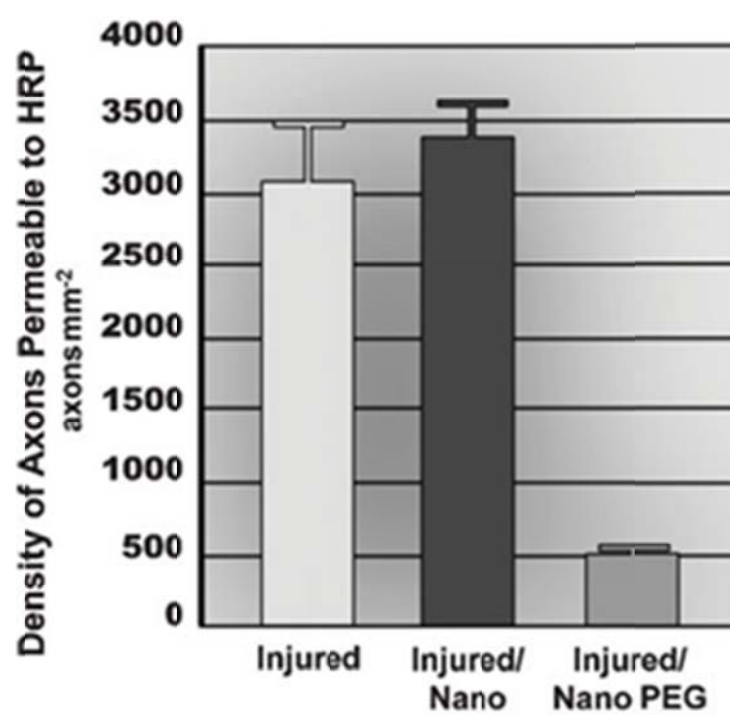


Figure 3.3 PEG Reduces Membrane Permeability. PEG was applied immediately after injury, and 15 minutes later the tissue was placed in an aqueous solution containing HRP for one hour. Axons with damaged membranes take up the intracellular marker HRP while sealed axons do not. PEG significantly reduces the density of axons permeable to HRP. The term “Nano” stands for Nano Dropper which is the manner in which PEG was introduced to the injury site. (Luo et al., 2002; Shi and Borgens, 2000; Koob et al., 2005)

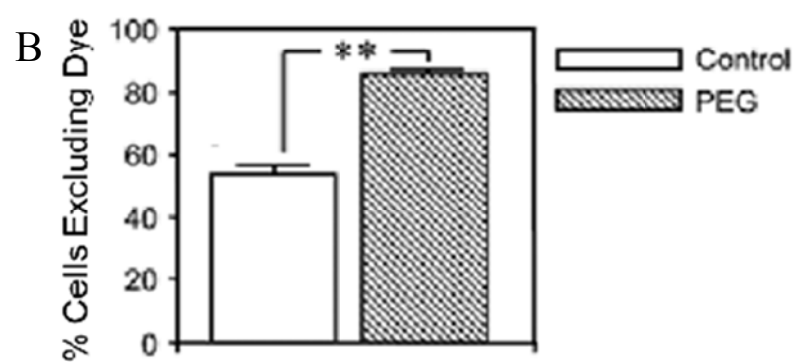
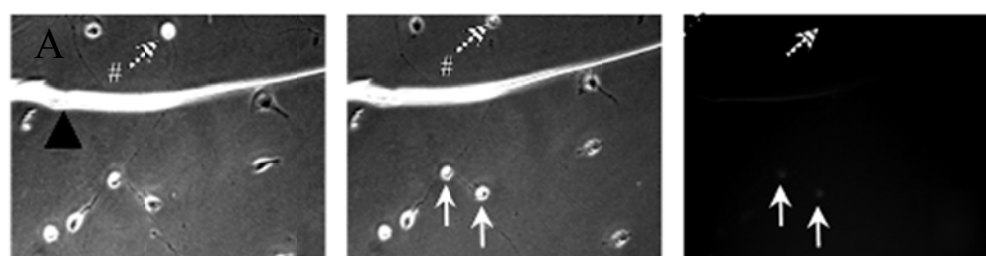


Figure 3.4 Transected Neurites Resealed by PEG. (A) These are DRGs grown in culture and their neurites cut indicated by the black triangle. Following the cut they are washed with PEG and at varying times after they are washed in a PBS soln containing the red dye TMR (tetramethylrhodamine) for 15 min. The cells are then rewashed 3 times to remove dye not taken up, and viewed under fluorescence, cells were determined to be sealed if their fluorescence was equal or less intense than neighboring, uninjured cells. The striped arrow indicates a DRG that resealed its injured membranes. (B) Graph indicating PEG enhanced membrane resealing. Figure modified from Nehrt et al., 2010.

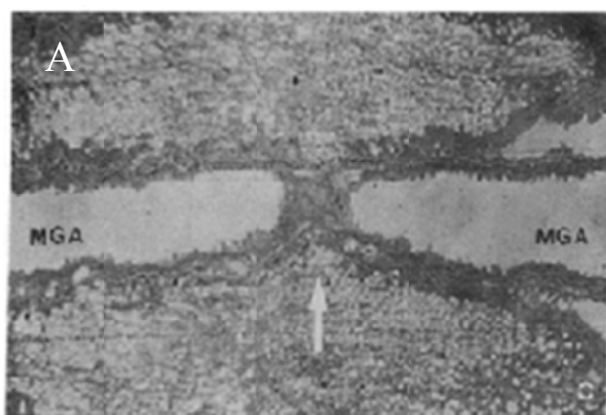


Figure 3.5 PEG Rapidly Fuses Closely Apposed Severed Axon Ends. Here the Medial Giant Axon (MGA) of a giant earthworm is cut. (A) As a control, saline is applied to closely apposed cut axons, however no resealing occurs. (B) When PEG is applied to closely apposed cut ends, rapid resealing occurs, and within 60 seconds greater than 80 percent of trials resulted in fusion (Krause & Bittner, 1990).

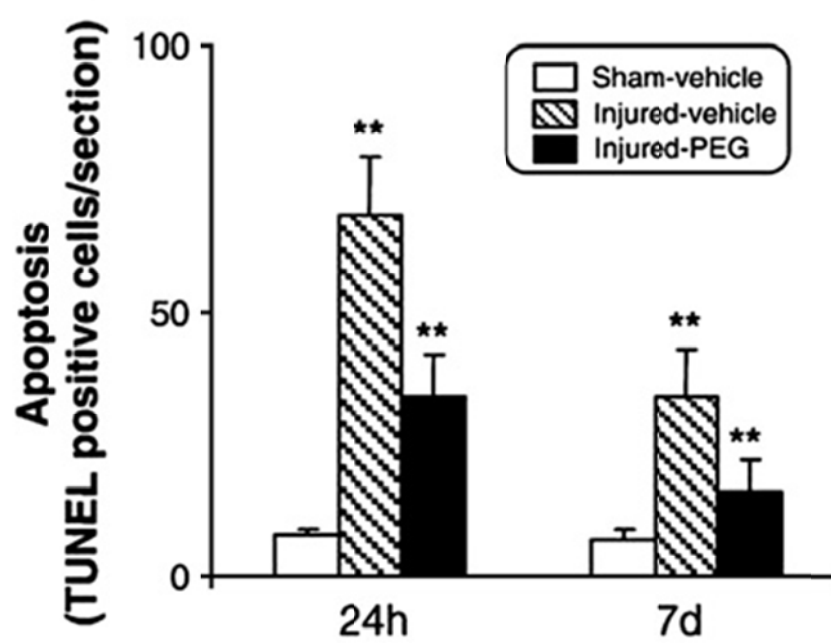


Figure 3.6 PEG Reduces Apoptosis in Injured Neurons. Shown here is TUNEL assays run after a compression injury at T10-T11. Compression injury induced a significant increase in apoptosis at 24 hours and 7 days post injury with a higher value at 24 hours after injury. Treatment with PEG immediately after injury significantly reduced apoptosis in the epicenter of the injury site at both 1 day and 7 days after injury. This is likely related to PEG quickly sealing damaged membranes and limiting the ionic disruption that can activate apoptosis. $**P < 0.01$ between sham-vehicle and injured-vehicle or injured-vehicle and injured-PEG groups. Figure from Luo & Shi, 2007.

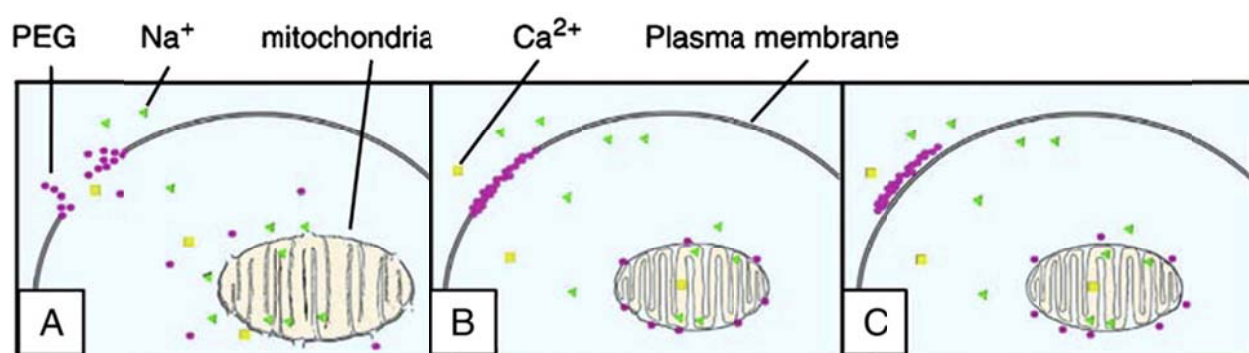


Figure 3.7 Hypothesis of Possible Mechanisms for PEG-Mediated Neuroprotection. (A) A traumatic injury to the spinal cord causes breaches in neuronal membrane and damages to cytosolic organelles such as mitochondria. Extracellular molecules enter the injured cell through the damaged membrane. When PEG is applied, some of the PEG molecules work to repair the membrane, while at the same time some PEG molecules enter the cytosol. (B) PEG forms a protective film spanning the membrane breach, dehydrates the local membrane areas, and facilitates the movement of components of the membrane adjacent to the breach. This also prevents the continued entry of extracellular molecules. PEG within the cytosol reaches mitochondria, protecting them from swelling. (C) The components of the membrane adjacent to the breach flow into one another and membrane breach is sealed. Mitochondrial swelling is reduced. Figure from Luo & Shi, 2007.

Chapter 4:

Assessing the Role of PEG Following a Transectional Spinal Cord Injury

Introduction:

There are numerous challenges to overcome in the course of treating spinal cord injury. Following injury, axons are traumatically injured if not completely severed, and in the majority of spinal cord injuries a gliotic scar forms around the damaged tissue often creating a fluid filled cyst. The scar is made of reactive cells that inhibit axon regeneration, and the fluid filled cyst is a physical gap regenerating axons are unable to cross. A multifaceted, combinatorial approach to treating SCI is necessary. We have addressed these issues using our air-gap electrospinning technique to create a three dimensional highly aligned bridge to implant in to the lesion area. Our substrate is polydioxanone suture (PDS) which is a biocompatible, resorbable suture that breaks down over time in the body. We previously demonstrated the ability of our bridge to support and direct neurite growth, and hypothesize that using PEG, a membrane fusogen, to rapidly seal damaged membranes will reduce cell death following injury and facilitate recovery. This represents a novel combinatorial approach to treating SCI.

To test our hypothesis, we used a complete transaction injury model. Although a complete transection is clinically rare, it is the preferred laboratory model because the functional deficits are very reproducible and it allows for the implantation of our bridge. In a complete transection model it can also be concluded that any recovery is not the result of spared axons, which are present in an incomplete injury. Of the myriad of health problems experienced by SCI patients, impairment of motor function is the easiest to identify and chart for recovery. The open

field Basso, Beattie, and Bresnahan, or BBB, locomotor test rates the effectiveness of hindlimb movements (Basso et al., 1995). BBB scoring ranges from 0 to 21 with 0 being complete paralysis and 21 being complete mobility. Scoring measures joint movement, limb coordination, gait stability, and weight support. We used the metric of hindlimb mobility to show that animals that received a PDS bridge saturated in PEG recovered better than animals that received a bridge saturated in saline.

Results:

Behavioral Assessment:

In our experiments, we are adding PEG, a pharmacological agent, to our bridge. During the weeks following surgery, behavioral recovery is our only metric to determine the efficacy of PEG. Using the BBB scoring system, we documented the progressive recovery of each animal following surgery. Figure 4.1 contains graphs displaying the average BBB score of the animals receiving a saline soaked bridge and animals receiving a PEG soaked bridge. The PEG animals had a greater average BBB score at both one and two months following injury. In Figure 4.2, the individual weekly BBB scores are documented and charted over the course of 1 month. The animals treated with a saline soaked bridge displayed virtually no functional recovery over a 4 week period. However, the PEG animals have a wide variability in their scores with some showing remarkable progress over a short period. Over the course of 2 months, the animals that received a bridge without PEG showed modest improvement, but only in the second month did these control animals begin to show progress (Figure 4.3). The PEG animals over a 2 month period showed a wide range of improvements with some animals recovering to near uninjured levels (Figure 4.3). Using functional recovery as our metric, the addition of PEG to our bridges

appears to have increased axon survival and/or regeneration following a traumatic spinal cord injury.

Histology:

To examine the histology of our animals we sectioned the lesion area of the spinal cords into 100 micron thick sections on a vibratome and processed them for electron microscopy. When compared with an uninjured animal (Figure 4.4), injured animals from both the saline and the PEG groups suffered a severe loss of cytoarchitecture (Figures 4.5A and 4.5B). Figure 4.5A is the lesion of a one month survival animal that received a saline soaked bridge. There is no discernable cytoarchitecture, and what appears to be an ongoing inflammatory response. Infiltration of fibroblasts has allowed for the formation of scar tissue, and while there are a few myelinated axons, the number seen is not near the number of the animals that received a PEG bridge (Figure 4.6). Figure 4.5B is the lesion of a one month survival animal that received a PEG soaked matrix. There is a similar inflammatory response and scar tissue pattern. However, upon closer examination, some organization and healthy axons can be seen on what appears to be the ventral portion of spinal cord (Figure 4.7). Similar results are seen in the two month survival group. While there is significant tissue loss and a lack of organization healthy axons can be seen in the ventral aspect of the spinal cord in the PEG group (Figure 4.8 and 4.9). Based on the histology, it appears that the addition of PEG to our bridge may be preferentially protecting ventral axons and promoting their survival and regeneration.

When comparing the histology of the PEG animals to an uninjured thoracic cord it is clear at 1 month the ventral region of the spinal cord of the PEG animals has been damaged (Figure 4.10). In the injured cord, degenerating axons can be seen as well as gaps in between

healthy axons, however, in the uninjured cord only a dense region of healthy axons is seen.

Figure 4.11 is a comparison of an uninjured animal with a 2 month PEG animal, and the increased recovery time appears to have allowed more axons to regenerate. This correlates with our increase in functional recovery and BBB scores as the PEG animals continued to progress during the second month of recovery.

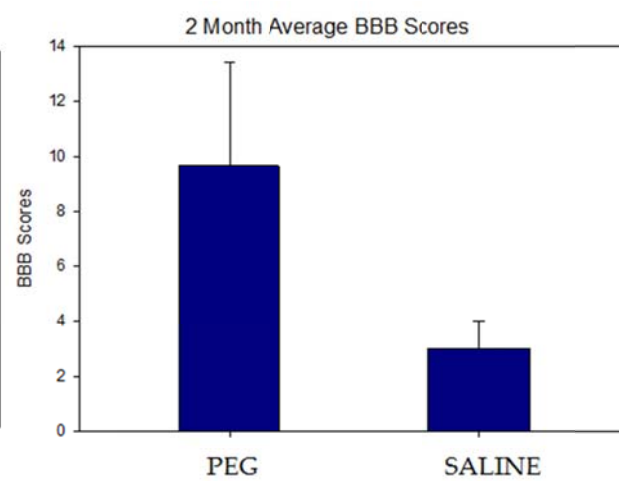
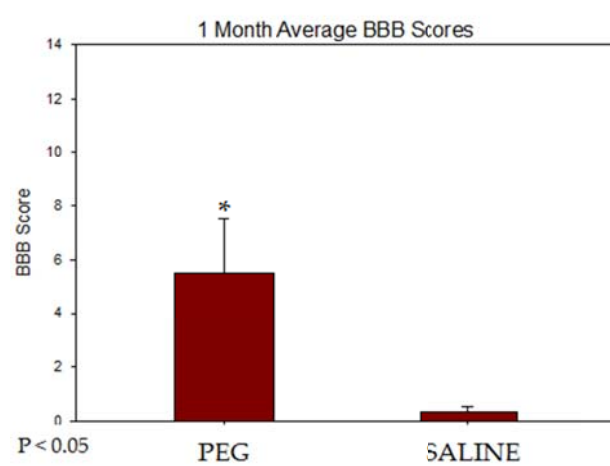


Figure 4.1 Average BBB Scores. Over the course of our trials, we measured hindlimb mobility using the BBB scoring system. Here are the average scores over the 1 month and 2 month survival periods. n=6 for the 1 month groups and n=3 for the 2 month groups. There is an obvious difference in scoring indicating that PEG may be improving functional recovery following SCI. *P < 0.05 between 1 month PEG and 1 month saline. The range in BBB scores in the 2 month PEG group was too great to be statistically significant when compared to the 2 month saline group.

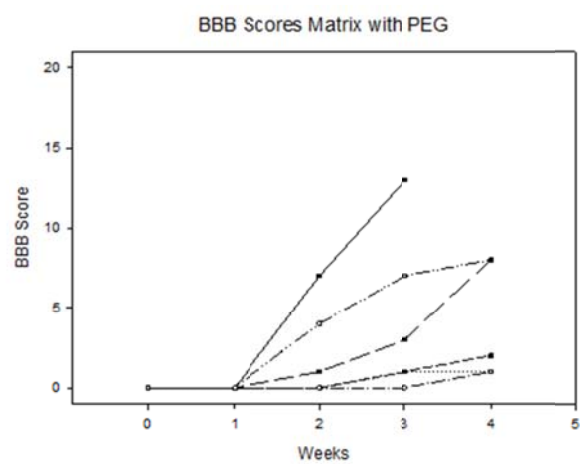
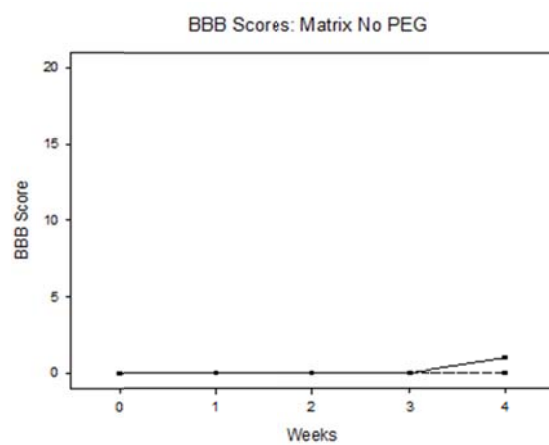


Figure 4.2 Weekly BBB Scores Over 1 Months. Animals without PEG in their implanted bridge showed no progress over a 4 week time period. Animals with PEG displayed various amounts of recovery over a 4 week period with some animals having significant improvements.
n=6

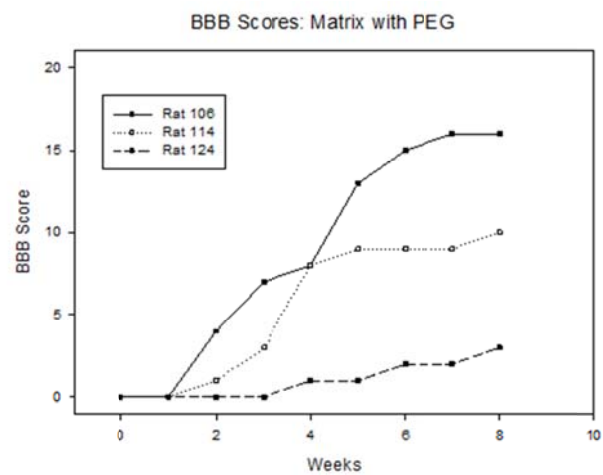
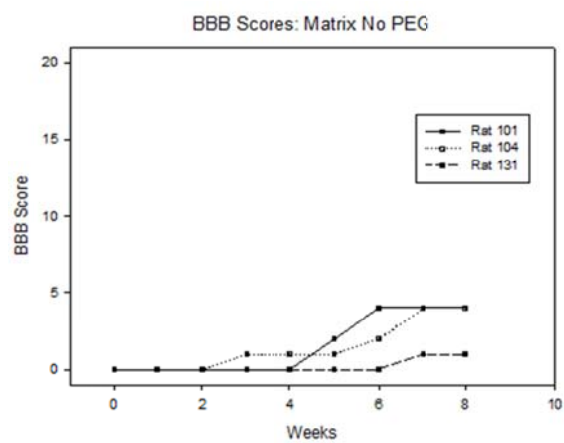


Figure 4.3 Weekly BBB Scores Over 2 Months. Animals without PEG in their implanted bridge showed only limited recovery over an 8 week time period, and did not progress until after 4 weeks. Animals with PEG displayed various amounts of improvement over an 8 week period with some animals making weekly progress and recovering to near uninjured levels. n=3

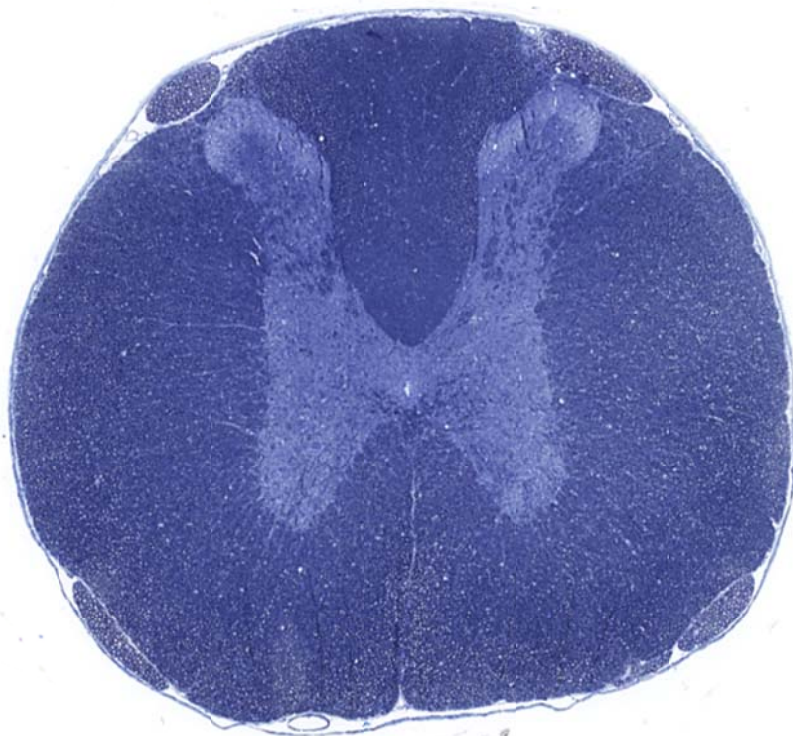


Figure 4.4 Coronal Section of Uninjured Rat Spinal Cord. This is an image of a thoracic semi-thin section of an uninjured rat spinal cord. The cord is oriented with the dorsal end up. Notice the uniform cytoarchitecture of the inner grey matter containing cell bodies and the outer white matter containing axon tracts.

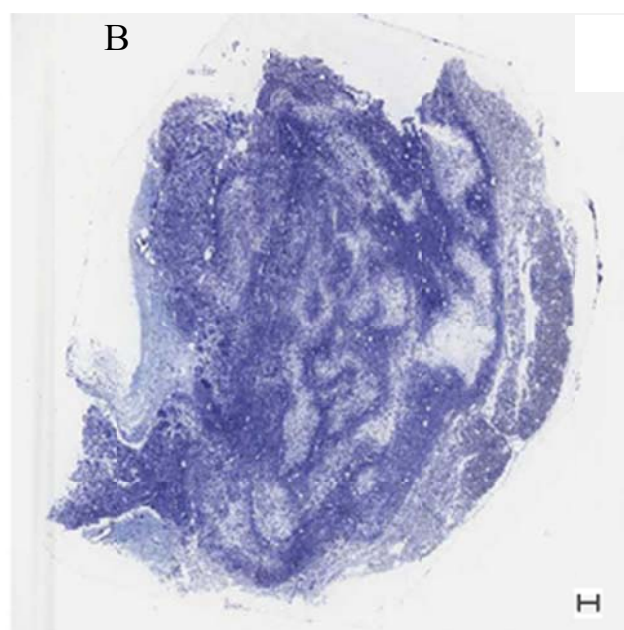
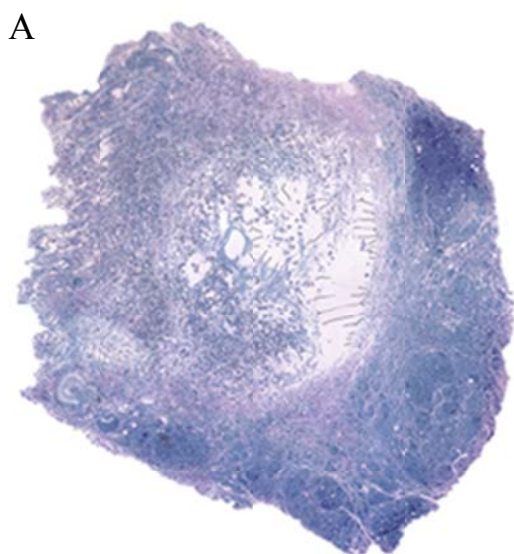


Figure 4.5 Semi-Thin Sections of 1 Month Lesion. (A) Semi-thin image of an animal that received a saline saturated bridge. There is complete loss of cytoarchitecture, and the loss of tissue in the center of the section is evidence of the formation of fluid filled cyst. (B) Semi-thin of an animal that received a PEG saturated bridge. There is a significant loss of cytoarchitecture however there isn't the appearance of a fluid filled cyst. The ventral aspect of the cord seems to have retained some organization and upon greater magnification large caliber myelinated axons can be seen. Scale bar for both images = 100um.

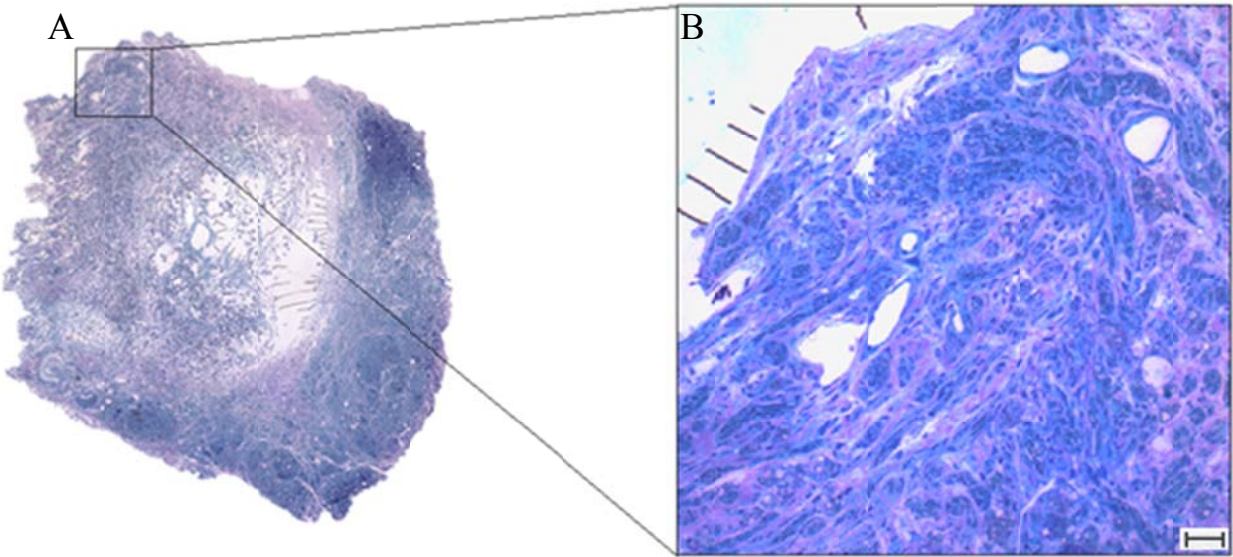


Figure 4.6 Images of 1 Month Saline Animal. (A) Expanded view of semi-thin section showing scar tissue formation and cavitation (B) Zoomed image showing a sparse number of small caliber myelinated axons surrounded by scar tissue. Scale bar = 40um.

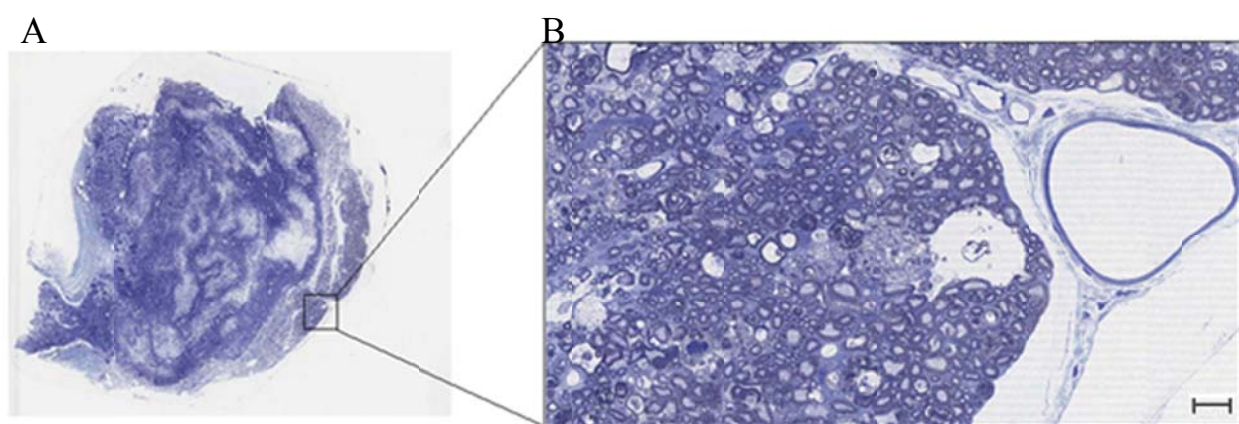


Figure 4.7 Images 1 Month PEG Animal. (A) Expanded view of semi-thin section showing scar tissue formation and disruption of cytoarchitecture (B) Image of healthy axons in the ventral region of the cord. Scale bar = 10um

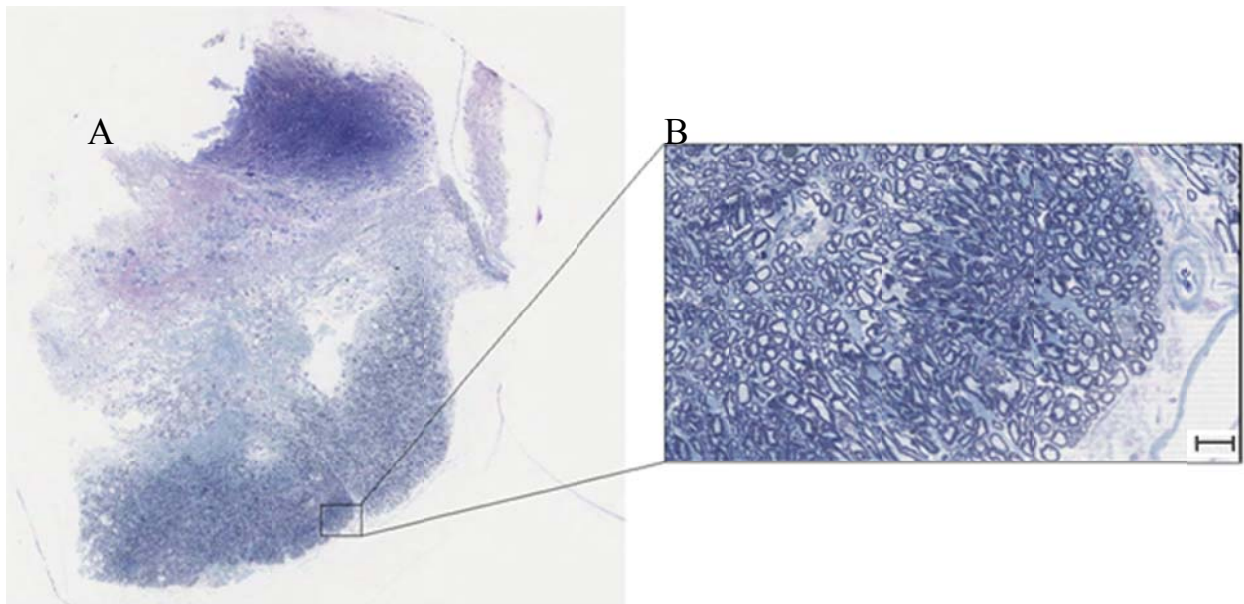


Figure 4.8 Images of Surviving Axons in 2 Month PEG Animal. (A) Expanded view of a semi-thin section displaying significant tissue loss, but some minor organization in the ventral region. (B) Image of healthy axons in what appears to be the ventral region of the cord. Scale bar = 10um.

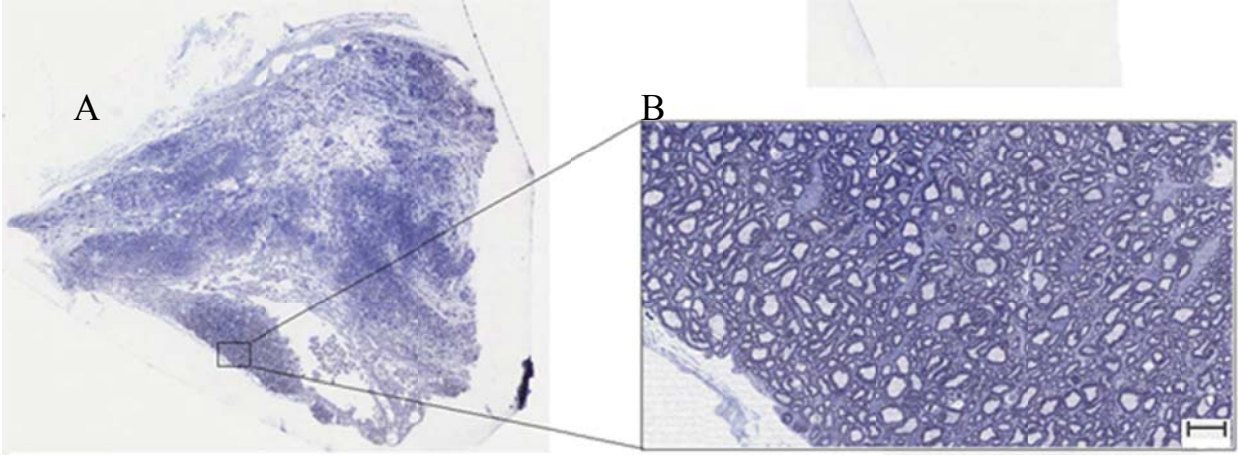


Figure 4.9 Images of Surviving Axons in 2 Month PEG Animal. (A) Expanded view of a semi-thin section displaying significant tissue loss and complete disruption of cytoarchitecture. (B) Image of healthy axons, however there are not any markers to indicate where in the spinal cord these axons are. Scale bar = 10um.

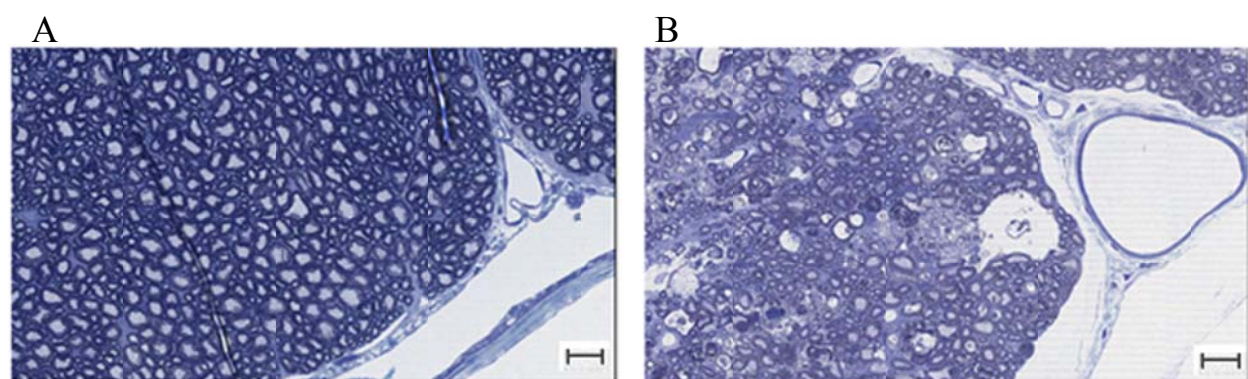


Figure 4.10 Ventral Comparison of Uninjured Cord and 1 Month PEG. (A) Image of the ventral region of an uninjured animal. (B) Image of an animal that received a PEG soaked bridge 1 month after injury. There are surprising similarities in overall structure. The injured animal has a less dense network of healthy axons indicating some damage occurred. Scale bar for both images = 10um.

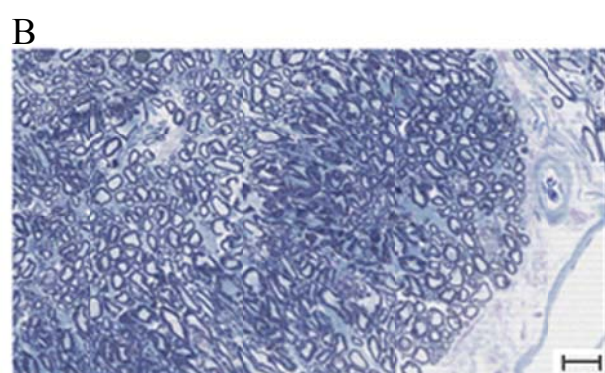
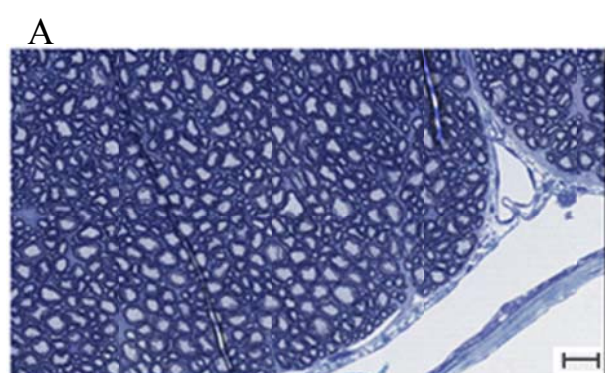


Figure 4.11 Ventral Comparison of Uninjured Cord and 2 Month PEG. (A) Image of the ventral region of an uninjured animal. (B) Image of an animal that received a PEG soaked bridge 2 month after injury. There are more similarities here than in Figure 4.10. The 2 months of recovery seem to have allowed for a greater density of axons to regenerate. Scale bar for both images = 10um.

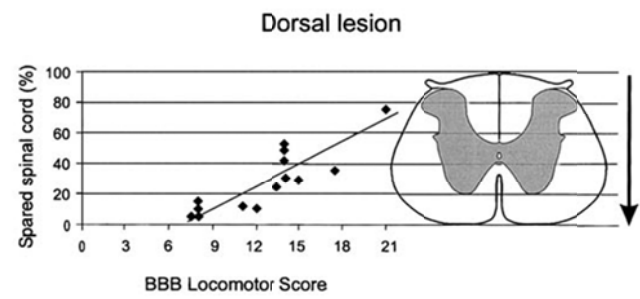
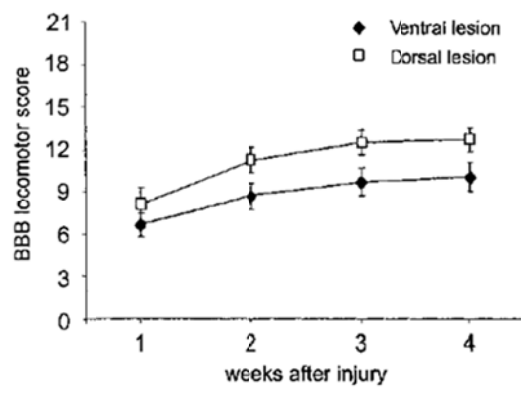


Figure 4.12 Hemisection Lesions and BBB Scores. (A) Following dorsal or ventral hemisection lesions, injured animals do not experience complete paralysis based on their BBB scores which consistently remain above 6. (B) BBB score decreases with depth of dorsal hemisection, however, BBB scores do not drop to 0. Figure from Fouad et al. Exp Neurol. 2002

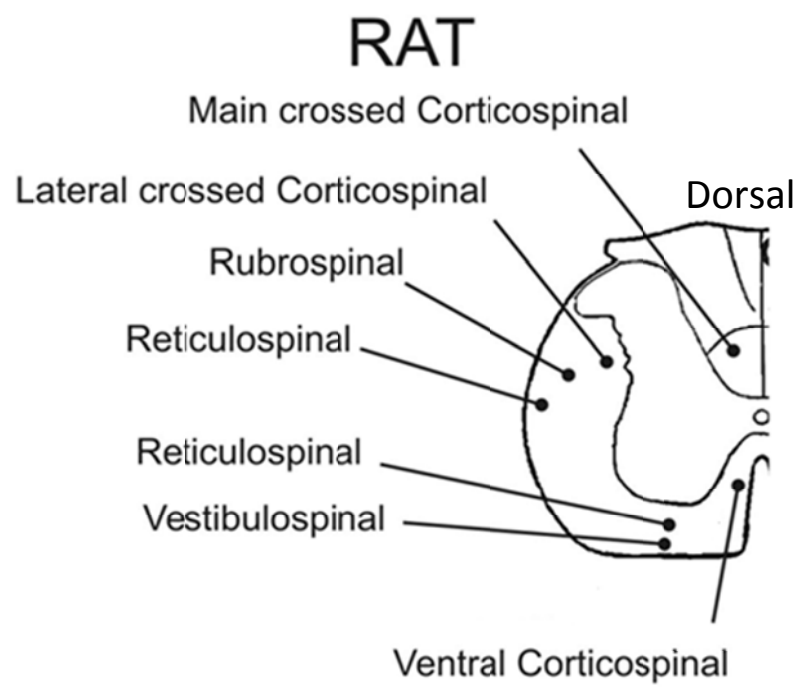


Figure 4.13 Axon Tracts Facilitating Lower Limb Movement. The ventral rat spinal cord contains axons tracts crucial in lower limb movement and locomotion. Corticospinal: fine motor control of limbs and digits (less involved in locomotion) Vestibulospinal: Locomotion (walking, trotting, galloping) Reticulospinal: Excitatory to extensor motor neurons, Locomotion initiation (stepping) and posture. Figure from (Majczyński & Sławińska, 2007)

Discussion

Functional Recovery:

Our primary metric for determining the success of our hypothesis in the weeks following surgery is hindlimb mobility. We discussed in chapter 3 the ability of PEG to fuse damaged membranes and decrease apoptosis, and our behavior results appear to confirm membrane resealing and neuron survival. By the second week following injury, many of the animals treated with PEG had already shown some functional recovery, and by the fourth week all animals treated with PEG had some level of functional recovery with a BBB score above 0. In contrast, all the animals treated with saline displayed virtually no recovery by four weeks, and only limited recovery in the entire eight week time period. Using BBB scoring to assess the effectiveness of our PEG saturated bridge; it seems that PEG does increase locomotor recovery following a traumatic SCI. This recovery would lead us to conclude that more axons are surviving the traumatic injury following the application of PEG, and with more axons surviving there is an increase in functional recovery below the level of the lesion.

Histology:

In the histology, there appears to be more healthy axons in the animals treated with PEG, which would explain their high BBB scores. In both groups, there is a significant loss of tissue and disruption in the cytoarchitecture of the spinal cord. In the animals treated with PEG there is an increase in the number of healthy axons. Both groups show evidence of an inflammatory response as well as the deposition of scar tissue; however the histology reveals a ventral sparing of axons in the animals treated with PEG. The clustering of healthy myelinated axons in the

ventral region of the spinal cord was only found in the animals that demonstrated significant functional recovery greater than 10 points on the BBB hindlimb mobility scale.

Methodology:

With the tremendous success of some but not all of the animals receiving a PEG soaked implant, it forces our lab to question the methodology of our surgeries. The histology showed a ventral sparing of axons. There may be a chance PEG preferentially protects the ventral cord, but the completeness of the transection also comes in to question. The surgery consisted of a single cut of the spinal cord with a pair of micro scissors following the thoracic laminectomy. Although care was made to ensure a complete transection, the most ventral region of the cord may have been missed. In studying the work of other scientists who use a dorsal hemisection model instead of a complete transection model an interesting difference was noticed. In the hemisection model where only the dorsal half of the spinal cord is transected, the BBB score of the injured animals never dropped to zero, and the animals never experienced complete paralysis (Figure 4.12). Following injury, the BBB score of all of our animals dropped to zero and remained there for a minimum of 7 days (Figure 4.2). In cases where hindlimb locomotion is being analyzed, if there is a small portion of ventral axon tracts that remain uninjured, the animals do not drop to a BBB score of 0 (Figure 4.12). Our behavioral data suggests if a complete transection was not performed, minimally all ventral axon tracts were damaged because all animals experienced complete paralysis for at least 7 days. PEG may be acting on these damaged ventral tracts, rapidly repairing them and allowing them to survive giving us the behavioral recovery we have seen.

Function of Ventral Spinal Tracts:

The ventral region in the PEG animals that appears to be either uninjured or repaired following injury contains major descending axon tracts that contribute to hindlimb motor function (Figure 4.13). Corticospinal, vestibulospinal, and reticulospinal axon tracts have projections that course through the ventral rat spinal cord. The corticospinal tract controls fine motor movements and is less involved in walking. The vestibulospinal tract plays a key role in walking and locomotion, as does the reticulospinal tract which is excitatory to extensor muscles and is important in initiating locomotion and maintaining posture (Majczyński & Sławińska, 2007). In the PEG animals the highest concentration of healthy axons was seen in the ventral spinal cord which explains their degree of functional recovery.

Limitations:

When dealing with such a traumatic injury and long survival period, it is challenging to create consistent results. It is very difficult to reproduce exactly what each animal experiences as each animal is different and they may react to the anesthesia, the implanted bridge, or the trauma of the injury in a different manner. Any animal model naturally produces some variability, but there are some steps we could take to reduce that variability.

In this study, the procedure was performed by two different surgeons who first had to learn the techniques. Previously, a neurosurgeon performed the surgeries, however, in recent studies a new surgeon had to be trained. In the future, the same surgeon should be used and should be well trained in order to provide a more consistent injury.

The electrospinning of our PDS bridges is another source of limitations. Spinning of the polydioxanone polymer is not always easy and be altered by changes in humidity and room

temperature. We are confident in the alignment of the bridge to give a direct path across the lesion site, and the scaffolds are cut to uniform thickness. However, the diameter of the 1mm discs is not always consistent. This issue needs to be addressed because a bridge smaller than the diameter of the spinal cord would not provide adequate support to crucial axon tracts along the periphery of the cord. Implanting an inadequate bridge does not help the animal or the science of spinal cord injury repair.

Presently we can't definitively say our success is a result of PEG sealing membranes, increasing cell survival, and promoting regeneration and recovery. It is, however, unlikely for an incomplete transection to be made by two different surgeons multiple times and only in the animals receiving a PEG saturated bridge. We believe PEG has a positive effect on cell survival, regeneration and recovery, but more experiments need to be done to determine the efficacy of the fusogen.

Future Directions:

Future experiments are aimed at reducing the variables associated with such a traumatic surgery and increasing the likelihood of regeneration and functional recovery. An experienced, consistent surgeon will perform the surgeries and minimize complications and trauma to the spinal cord and surrounding tissues. We would also like to maintain the integrity of the dura mater during and after the surgery. Closing the dura after a traumatic injury has been shown to increase functional recovery and reduce infiltration of cells such as fibroblasts that form scar tissue and should be excluded from the CNS (Iannotti et al., 2006). Future surgeries will determine if the dura mater can be sutured close or if a fibrin sealant works better. We will also consider reducing the injury size from a complete transection to a hemisection model. A

controlled hemisection would be less traumatic to the animal and allow us to focus on regeneration in a smaller area of the spinal cord. Future experiments will center on minimizing trauma to the animal, sealing the dura to protect the damaged cord, and possibly reducing the area of injury emphasizing regeneration of specific axon tracts.

In addition to improving the surgery, some modifications to the bridge are planned for the future. We believe PEG has a positive influence on the survival damaged neurons; however, PEG has not been shown to reduce inhibitors of regeneration. Future bridges will include PEG combined with Chondroitinase ABC (ChABC), an enzyme that breaks down chondroitin sulfate proteoglycans released by reactive astrocytes that inhibit axon regeneration. We also plan on adding nerve growth factor (NGF) in combination with PEG and ChABC. A bridge containing these three molecules would have the capacity to increase neuron survival with PEG, reduce inhibitory compounds with ChABC, and give an increased regenerative response with NGF.

The future of our histology will be directed toward experiments that can assess cell death. We will make comparisons of motor nuclei within the brainstem as well as the motor cortex of the rat brain. By comparing the brains of animals receiving the PEG treatment with control animals at the cellular level, we can assess the ability of PEG to increase neuron survival by looking at the neuron cell bodies.

Following improved surgical techniques implanting a modified bridge, future experiments will also focus on exercise rehabilitation. Exercise may be a crucial tool to maintain motor end plates and muscle tone, reduce weight gain, and increase regeneration from severed neurons. Several groups have reported increased locomotor recovery after SCI in rats and mice with the use of exercise therapy (Engesser-Cesar, Anderson, Basso, V R Edgerton, & Cotman,

2005; Giangregorio et al., 2005; Gómez-Pinilla, Ying, Roy, Molteni, & V Reggie Edgerton, 2002; Harkema, 2001; Multon, Franzen, Poirrier, Scholtes, & Schoenen, 2003; Raineteau, Fouad, Bareyre, & Schwab, 2002). Treadmill training for example has resulted in increased axonal sprouting proximal to the lesion, decreased muscle atrophy, and increased synapse formation (Goldshmit, Lythgo, Galea, Turnley, 2008).

In the future we hope to have the surgical experience necessary to perform a minimally invasive, reproducible injury that preserves the dura. Our bridge will have fusogenic capabilities as well as neurotrophic support promoting axon growth. Exercise rehabilitation will increase the survival of motor end plates as well as release growth factors and guidance molecules to facilitate axonal exit from the bridge. Successful treatment of traumatic spinal cord injury will take a combination of techniques and treatments, but the future of the field looks promising.

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Vita

Dustin Kelley Dalton was born in Monroe, North Carolina on July 20, 1986. He attended the University of North Carolina-Chapel Hill from 2004-2008 and received a Bachelors degree in Biology in 2008. He began his work towards a Master of Science in the Fall of 2009 at Virginia Commonwealth University.